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The fibroblast-specific MAb AS02: a novel tool for detection and elimination of human fibroblasts

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Abstract. The unwelcome presence of fibroblasts in many cell cultures prevents the long term cultivation of various cell types and work with pure populations. Recently, we described a novel fibroblast-specific monoclonal antibody (MAb AS02) that recognises a membrane-bound antigen. We have now developed a method using the fibroblast-specific MAb AS02 immobilised on goat-anti-mouse-magnetic beads to separate contaminating fibroblasts. An endothelial cell line experimentally contaminated with 5%–50% fibroblasts was successfully purified. Additionally, an endothelial cell line with an initial fibroblast contamination of 1.5% was prepared. A proportion of each preparation was cultured with no separation step being performed, whereas the remainder was cultured after purification with MAb AS02 to exclude the presence of a minor number of fibroblasts (<0.1%). The proportion of fibroblasts increased up to 38% in the fifth passage of culture without elimination of the low initial fibroblast contamination, whereas in the fraction with the separation step, no fibroblasts were detectable by flow cytometry, even after the fifth passage. We also used the antibody to detect the presence of naturally contaminating fibroblasts in thyrocyte cultures. After cultivation of thyrocyte cultures over five passages, the number of fibroblasts increased dramatically up to 50%–80% of the whole population. Subsequently, we successfully applied the method for complete elimination of naturally contaminating fibroblasts from freshly isolated thyrocyte cultures from enzymatically digested thyroid glands. Thus, MAb AS02 is a fibroblast-specific marker that is a useful tool for the detection and elimination of contaminating fibroblasts. The specificity of MAb AS02 permits the universal application of this antibody for human cell cultures of interest.

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Introduction

Cell culture is a common method for the investigation of cells from various tissues. However, the presence of fibroblasts in many cell cultures (epithelial cells, endothelial cells, chondrocytes and macrophages) and their high proliferative potential causes an overgrowth of cell cultures, even when the initial fibroblast contamination is low (Singer et al. 1989; Linge et al. 1989; Raj et al. 1984). To date, fibroblasts have been characterised mostly by their spindle-shaped morphology. Specific detection of these contaminants has often been difficult because of the lack of known appropriate markers. However, the detection of this contamination is essential for the correct interpretation of experimental results, especially when highly sensitive methods are used to measure cell products that are also produced by fibroblasts (Aust et al. 1996; Weetmann et al. 1990). Moreover, the overgrowth of cell cultures by fibroblasts also prevents the long term cultivation of many cell types. Several methods for the elimination of fibroblasts have been described (Halaban and Alfano 1984; Gilbert and Migeon 1975). Some of these methods are problematic, because of the non-specific destruction of cells or the requirement of repetitive application to reduce the amount of fibroblast contamination. For example, techniques involving the use of monoclonal antibodies have been described (Singer et al. 1989; Linge et al. 1989; Abboud et al. 1986); however, most of these monoclonal antibodies cross-react with the extracellular matrix (ECM; Esterre et al. 1992; Van Vliet et al. 1986) or with other cell types (Singer et al. 1989; Abboud et al. 1986; Esterre et al. 1992; Janin et al. 1990; Höyhtyä et al. 1984) and, therefore, have only a restricted use with certain cell cultures.

Recently, we have described two novel fibroblast-specific monoclonal antibodies (MAbs), AS01 and AS02,

that recognise membrane-bound antigens. In flow-cytometrical and immunohistochemical studies, we have demonstrated that the antibodies react only with human fibroblasts. The antibodies do not react with fibroblasts from rat, mouse or pig (Saalbach et al. 1996). In the present study, we report the detection and elimination of human fibroblasts in experimentally (endothelial cells/fibroblasts) and naturally (thyrocyte cultures) contaminated cell cultures, when using the fibroblast-specific MAb AS02.

Materials and methods

Cell types

Thyrocytes. Thyroid tissue was trimmed of fat and connective tissue immediately after surgery. Thyrocytes were enriched after gradual enzymatic digestion of tissue as described (Davies 1985). Briefly, the thyroid cell suspension resulting from mechanical disaggregation followed by enzymatic digestion (5×30 min, change of supernatant after every 30 min) with dispase (4.8 mg/ml, grade II; Boehringer Mannheim, Germany) was incubated for 18 h in complete culture medium (RPMI 1640; 10% fetal calf serum). Thyrocytes were obtained from the adherent fraction by incubation of the cell monolayer with phosphate-buffered saline (PBS; without Ca^{2+}/Mg^{2+}) for 45 min. The cells were passaged once a week.

Fibroblasts. These were obtained after outgrowth from skin biopsies. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM-Glutamax; Gibco, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Gibco), 50 mg/l ascorbic acid, 50 µg/ml streptomycin and 400 U/ml penicillin and passaged after reaching confluence by using 0.05% trypsin, 0.02% EDTA (Gibco).

Transformed human dermal microvascular endothelial cell line HMEC-1. (Ades et al. 1992; kindly provided by Dr. T. Lawley). These cells were cultured in EBM (Endothelial cell basal medium) with supplements (Clonetics, USA) and 5% fetal calf serum and passaged after reaching confluence (every 3–4 days) by using 0.025% trypsin, 0.02% EDTA (Clonetics, USA).

Immunohistochemistry

Cells were cultivated on glass coverslips, washed with PBS, fixed with methanol for 10 min and dried. Samples were incubated for 1 h with unpurified hybridoma culture supernatant (undiluted; approximately 30 µg/ml) or an anti-thyroid-peroxidase (TPO) antibody as a marker for thyrocytes (MAb A4 was a gift from Dr. P. Banga, King's College School of Medicine, London) followed by incubation with goat-anti-mouse antibody (Dako, Carpenteria, Calif.) and APAAP complex (Dako). Washing was performed with 0.9% NaCl/0.05 M TRIS-HCl, pH 7.4. Bound antibodies were detected by the New Fuchsin Substrate System (Dako). Counterstaining was performed with haematoxylin.

Flow cytometry

Cell monolayers were detached by 0.05% trypsin, 0.02% EDTA (Gibco) and washed twice with PBS. Cells (2×10^5) were incubated with 50 µl hybridoma culture supernatant containing MAb AS02 (undiluted; approximately 30 µg/ml) as the fibroblast marker and anti-CD31 MAb (5 µg/2×10^5 cells; Immunotech, Hamburg, Germany) as the endothelial cell marker, for 45 min at 4° C, or an irrelevant isotype matched MAb as a negative control (background staining). After being washed with PBS/10% Gelafusal, the cells were incubated for 45 min at 4° C with fluorescein-isothiocyanate-conjugated goat-anti-mouse antibody, washed three times and fixed with PBS/10% Gelafusal/1% formaldehyde. The final evaluation was performed by using flow cytometry in an EPICS-Flow cytometer (Coulter, Krefeld, Germany).

Coupling of MAb AS02 to goat-anti-mouse magnetic beads

Goat-anti-mouse, IgG-DYNABEADS M450 (Dynal; Hamburg, Germany) were washed three times with PBS/0.1% bovine serum albumin. Subsequently, 4×10^8 magnetic beads were incubated with 1 ml culture supernatant of the fibroblast-specific MAb AS02 in concentrated form (~20 µg MAb) overnight at 4° C. Beads washing was repeated after coupling and the conjugates were used for cell separation. There were no differences of separation efficiency by using either beads labelled with MAb AS02 or cells labelled with the MAb.

Removal of fibroblasts

Single-cell suspensions of experimentally mixed endothelial cells and fibroblasts or thyrocytes with a natural contamination of fibroblasts were prepared and washed with PBS. To eliminate fibroblasts, 0.5 ml cell suspension was incubated with MAb AS02 coupled to magnetic beads, with gentle shaking for 1 h at room temperature. The best results were obtained when 40 beads were used per cell (cell:beads ratio of 1:40). The AS02-positive cells were separated with a magnet. The remaining cells were analysed for the presence of fibroblasts by flow cytometry or immunohistochemistry, as described, or were subsequently sub-cultured.

Results and discussion

The overgrowth of many cell cultures by fibroblasts is a well-known problem (Singer et al. 1989; Linge et al. 1989; Aust et al. 1996). In recent studies we have shown

Table 1. Reactivity of MAb AS02 in human tissue[a]

Connective tissue		Kidney	
Fibroblasts	+	Glomeruli	±[b]
Fat cells	–	Tubulus epithelium	±
Endothelial cells	–	Fibroblasts	+
Cells, glandular	–		
Chondrocytes	–	Lymphatic tissue	
		Lymphocytes	–
Muscle		Fibroblasts	+
Fibroblasts	+		
Smooth muscle	–	Placenta	
Skeletal muscle fibres	–	Mesenchyme	+
		Epithelium	–
Umbilical cord		Endothelium	–
Endothelium	–		
		ECM proteins[c]	
Liver/gall bladder		Collagen I, III, IV	–
Epithelium	–	Fibronectin	–
Endothelium	–	Laminin	–
Parenchyme	–		
Fibroblasts	+		

[a] Investigated by using immunohistochemical techniques (Saalbach et al. 1996)
[b] Occasional cells were stained
[c] The binding of MAb AS02 to ECM proteins was examined by ELISA (Saalbach et al. 1996)

Table 2. Proportion of fibroblasts (%±SEM) in an experimentally mixed endothelial cell/fibroblast population before and after separation with various ratios of cells to MAb AS02-magnetic-bead conjugates[a]

Before separation	Ratio of cells to magnetic beads			
	1:5	1:10	1:20	1:40
Low contamination AS02⁺: 10±2% CD31⁺: 90±1.5% Negative control: 0.1%	0.1%[b]	0.1%	0.1%	0.1%
High contamination AS02⁺: 50±5% CD31⁺: 50±5% Negative control: 0.2%	1.4±0.3%	0.4±0.2%	0.3±0.2%	0.1%

[a] The cell population was analysed before and after the separation by flow cytometry with MAb AS02 to defect fibroblasts; anti-CD31 for endothelial cells and an isotype matched antibody as negative control

[b] Percent AS02⁺ cells after separation (mean±SEM); 0.2% equals the background staining in flow cytometry

that MAb AS02 binds to human fibroblasts from a variety of tissues (skin and connective tissue of placenta, kidney, liver, muscle, lymph node and gall bladder). We have found no reactivity with epithelial cells, blood cells, muscle or the ECM by immunohistochemistry, flow cytometry or enzyme-linked immunosorbent assay (ELISA; Table 1). The specificity of MAb AS02 to fibroblasts and the recognition of fibroblasts of different tissue origin by MAb AS02 enable the use of this MAb for the detection and separation of contaminating fibroblasts from various human cell culture systems. Double-staining experiments with an anti-proline-4-hydroxylase antibody have shown that MAb AS02 reacts with all fibroblasts in vitro and in situ (data not shown). In this study, we thus report the application of the fibroblast-specific MAb AS02 (Saalbach et al. 1996) for the detection and elimination of fibroblasts from experimentally and naturally contaminated cell cultures.

Elimination of fibroblasts from an experimentally mixed population of endothelial cells (HMEC-1) and fibroblasts

We used the MAb AS02 immobilised on magnetic beads for the binding and separation of fibroblasts from experimentally mixed endothelial cell/fibroblast populations that were used as an experimental model for optimising the method. First, we optimised the necessary ratio of cells to MAb AS02-magnetic-bead conjugate for complete elimination of the fibroblasts from endothelial cell/fibroblast mixtures. Two suspensions, one with a high content of fibroblasts (50%) and the other with 10% fibroblasts, were prepared. Ratios of cells:beads of 1:5, 1:10, 1:20 and 1:40 were chosen. After separation

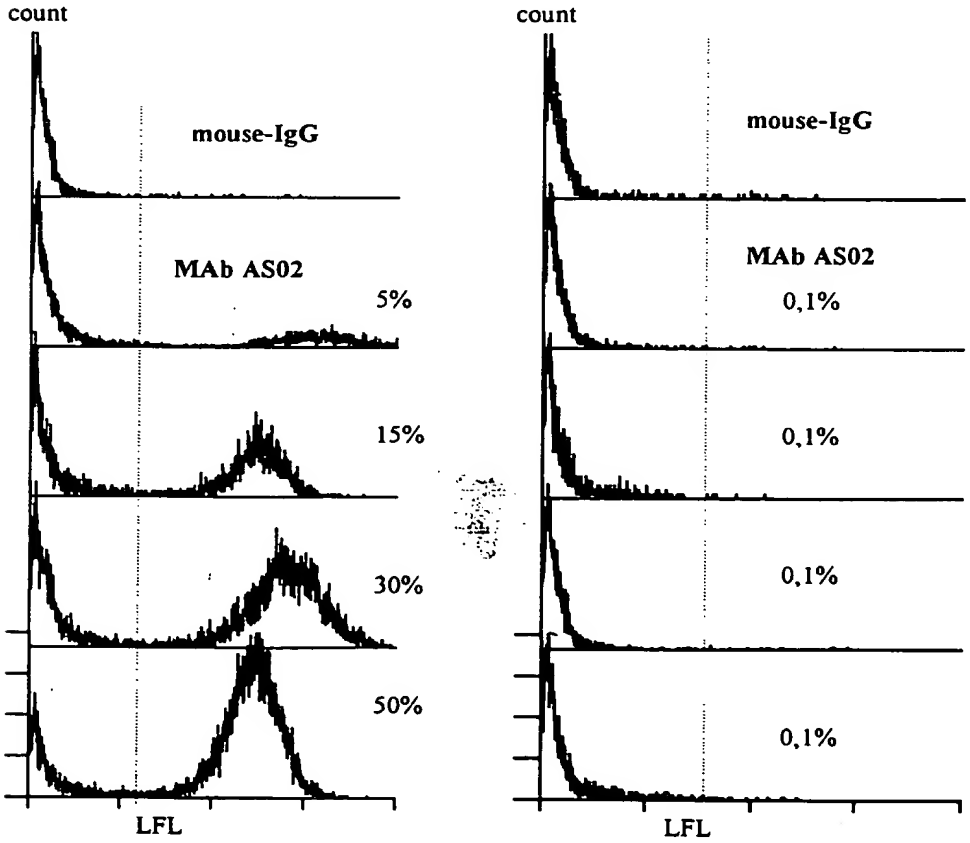
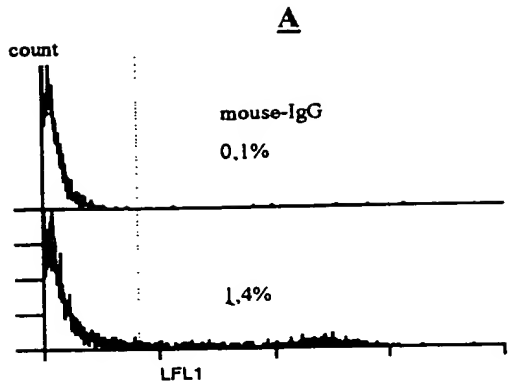


Fig. 1. Elimination of fibroblasts from an experimentally mixed endothelial cell/fibroblast population. Endothelial cells were mixed with fibroblasts to obtain populations with 5%, 15%, 30% and 50% fibroblasts. The fibroblasts were eliminated with magnetic-bead-MAb AS02 conjugate by using a cell:bead ratio of 1:40. Before (*left*) and after (*right*) separation, the fibroblasts were detected by staining with MAb AS02 and flow-cytometric evaluation. *LFL*: Log fluorescence



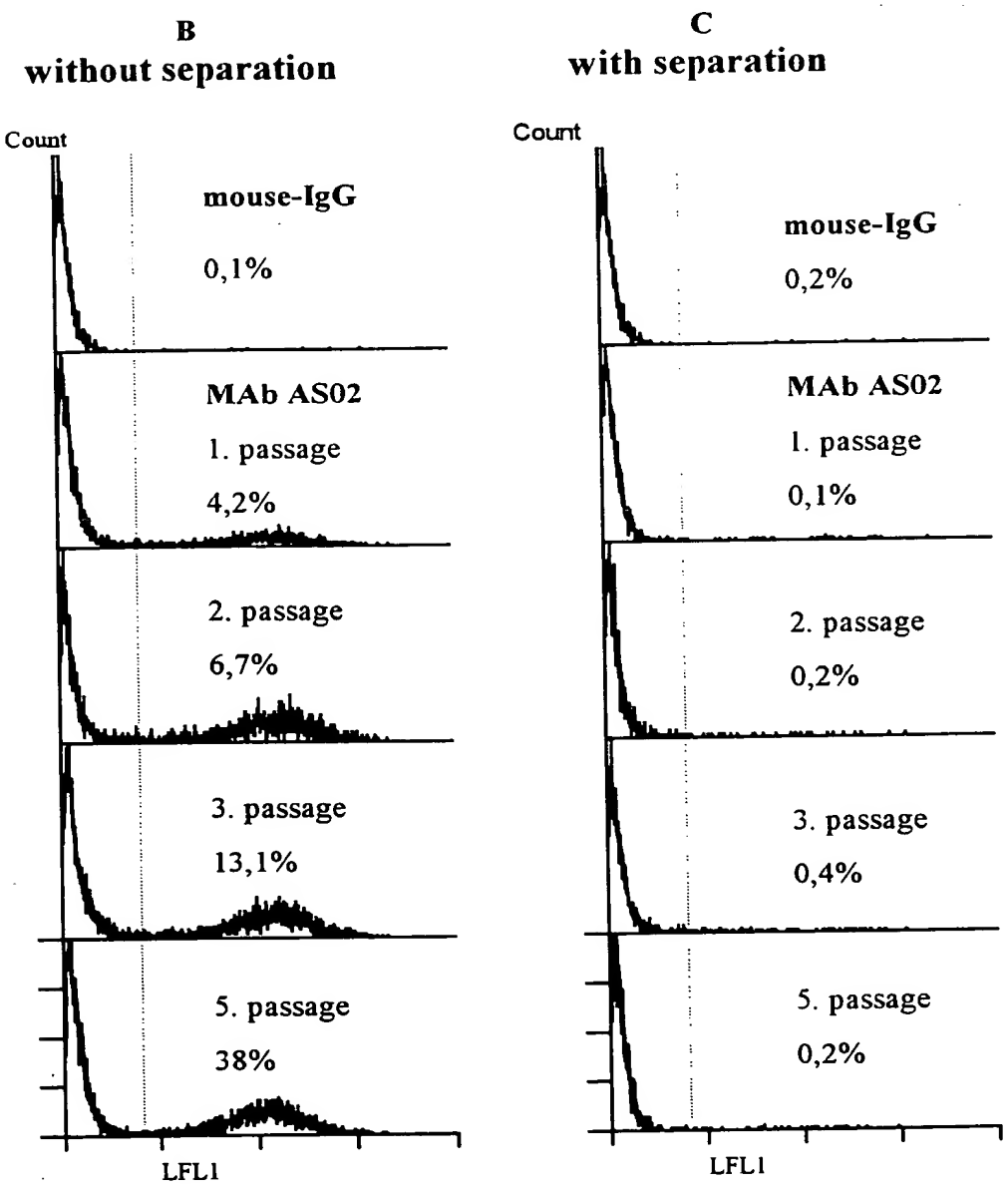


Fig. 2A–C. Analysis of fibroblast contamination after a long cultivation period of an experimentally mixed endothelial cell/fibroblast population with no fibroblast elimination or with separation of fibroblasts by using the MAb AS02-magnetic-bead conjugate. Endothelial cells (HMEC-1) were mixed with 1.5% fibroblasts (**A**). A proportion of these were treated with MAb AS02 conjugated to magnetic beads (**C**), whereas the remainder were not treated (**B**). Both populations were cultivated up to the fifth passage. The level of fibroblast contamination after every passage was detected by flow cytometry by using MAb AS02. A mouse IgG isotype control antibody was also used (background staining). *LFL1:* Log fluorescence

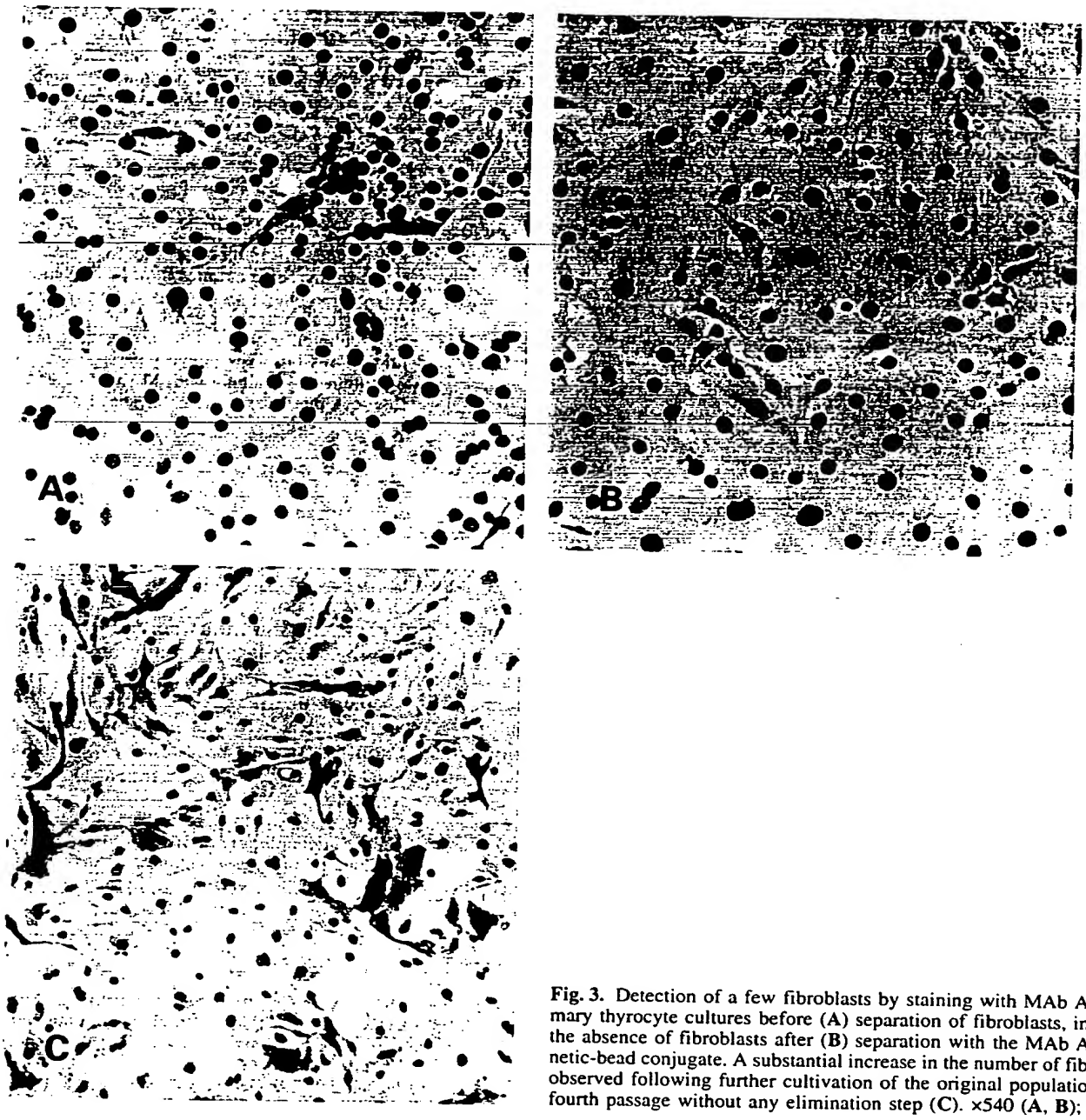


Fig. 3. Detection of a few fibroblasts by staining with MAb AS02 in primary thyrocyte cultures before (**A**) separation of fibroblasts, in contrast to the absence of fibroblasts after (**B**) separation with the MAb AS02-magnetic-bead conjugate. A substantial increase in the number of fibroblasts was observed following further cultivation of the original population up to the fourth passage without any elimination step (**C**). ×540 (**A**, **B**); ×270 (**C**)

of the population contaminated with 10% fibroblasts, no fibroblasts were detectable by flow cytometry at any of the tested ratios. However, when the cell population was contaminated with 50% fibroblasts, a ratio of 1:20–1:40 was necessary to eliminate all fibroblasts (Table 2).

We also investigated the capacity of this method to eliminate different proportions of fibroblast contamination (5%, 15%, 30% and 50%) from an experimentally mixed endothelial cell/fibroblast population. After separation, no fibroblasts were detectable in the negative fraction by flow cytometry, in all cases (Fig. 1). Staining with propidium iodide did not show any loss of cell viability during the separation (data not shown).

The evaluation by flow cytometry did not however exclude the possibility that minor contaminating fibroblasts (<0.1% of the whole cell population) in the purified cultures may overgrow the target cells after longer cultivation periods. Therefore, an immortalised endothelial cell line (HMEC-1; Ades et al. 1992) with an initial fibroblast contamination of 1.5% was prepared. One part of the population was cultured with no separation steps whereas the remainder was purified as described above to eliminate fibroblasts. Both fractions were grown over five passages. After every passage, the populations were analysed for their content of fibroblasts by flow cytometry with MAb AS02. We demonstrated that, in purified cultures, no restored fibroblast contamination was detectable. In

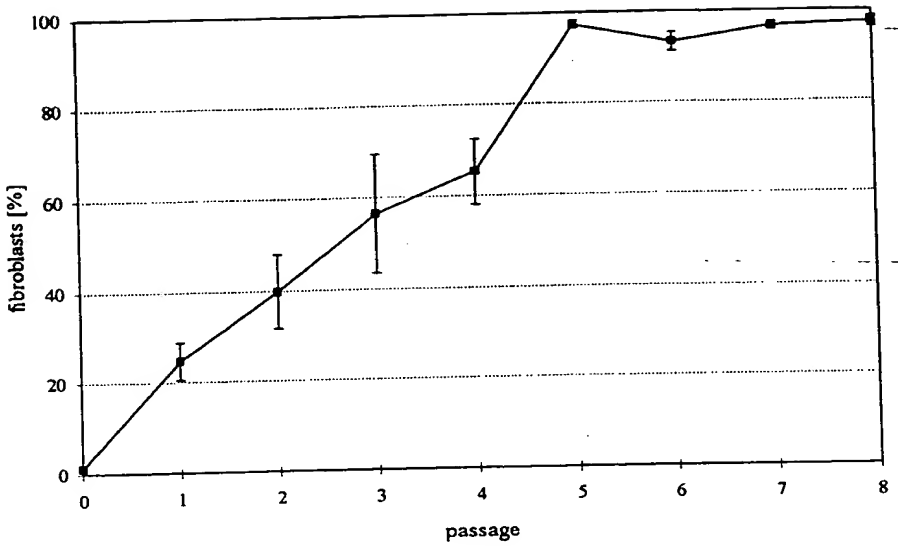


Fig. 4. Increase in the proportion of fibroblasts in thyrocyte cultures during cultivation. Thyrocyte cultures (n=4) were obtained from enzymatically digested thyroid glands and were cultured up to the 8th passage. The proportion of fibroblasts in every passage was determined by staining with MAb AS02 and flow-cytometric evaluation (mean±SEM)

contrast, even when initial fibroblast contamination was low (1%–2%) and despite the high proliferative potential of the endothelial cell line, the proportion of fibroblasts strongly increased up to 38% in the fifth passage in the contaminated cell culture without a prior separation step (Fig. 2A–C). The marked increase of fibroblasts over a short cultivation time (demonstrated for both endothelial cells and thyrocytes) makes it necessary to eliminate fibroblasts as early as possible in order to obtain a complete removal of fibroblasts. All experiments for optimisation were performed in triplicate. A representative analysis is shown in histograms of flow cytometry (Figs. 1, 2).

Elimination of naturally occurring fibroblasts in thyrocyte cultures

After optimising the method, we used MAb AS02 to detect and eliminate fibroblasts in thyrocyte cultures from enzymatically digested thyroid glands naturally contaminated with fibroblasts, as a practical application of the method. In the first passage of thyrocyte cell cultures, we found 20%–25% AS02-positive cells (fibroblasts) and about 80% TPO-positive cells (marker for thyrocytes). Immunohistochemistry allowed us to identity fibroblasts clearly with MAb AS02 in the cell cultures (fourth passage) independently of their morphology (Fig. 3C).

Furthermore, we analysed the level of fibroblast contamination in thyrocyte cultures during an extended cultivation period, by flow cytometry. In primary culture, the proportion of fibroblasts varied from 0%–2% of the total cell number. During cell cultivation, we observed an increase in the proportion of fibroblasts to 40%, even by the second passage, whereas fibroblasts completely overgrew the thyrocytes by the sixth passage (Fig. 4).

We used the described technique to remove fibroblasts from four different primary thyrocyte cultures with initial contamination of fibroblasts from 0.5%–2% of the whole cell number. After separation, the fibroblasts were completely eliminated from the cell culture as analysed by immunohistochemistry (Fig. 3A shows the staining of fibroblasts with MAb AS02 in thyrocyte cultures before separation, and Fig. 3B after separation) and by flow cytometry (data not shown).

The specificity of MAb AS02 thus permits its widespread use for detecting fibroblasts in many human cell cultures of interest, as demonstrated in this study with thyrocytes. After separation, the cells can be used for the analysis of specific mRNA by the reverse transcription/polymerase chain reaction or be cultured for longer periods of time (Aust et al. 1996).

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References

Abboud CN, Duerst RE, Frantz CN, Ryan DH, Liesveld JL, Brennan JK (1986) Lysis of human fibroblast colony-forming cells and endothelial cells by monoclonal antibody (6–19) and complement. Blood 68:1196–1200

Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ (1992) HMEC-1 establishment of an immortalized human microvacular endothelial cell line. J Invest Dermatol 99:683–690

Aust G, Heuer M, Laue S, Lehmann I, Hoffmann A, Heldin NE, Scherbaum WA (1996) Expression of TNF-a mRNA and protein in pathological thyroid tissue and carcinoma cell lines. Clin Exp Immunol 105:148–154

Davies TF (1985) Co-cultures of human thyroid monolayer cultures of human thyroid monolayer cells and autologous T cells – impact of HLA class II antigen expression. J Clin Endocrinol Metab 61:418–423

Esterre PH, Melin M, Serrar M, Grimaud JA (1992) New specific markers of human and mouse fibroblasts. Cell Mol Biol 38:297–301

Gilbert SF, Migeon BR (1975) D-valine as a selective agent for normal human and rodent epithelial cells in culture. Cell 5:11–17

Halaban R, Alfano FD (1984) Selective elimination of fibroblasts from cultures of normal human melanocytes. In vitro 20:447–450

Höyhtyä M, Myllylä R, Piuva J, Kivirikko K, Tryggvason K (1984) Monoclonal antibodies to human prolyl-4-hydroxylase. Eur J Biochem 141:477–482

Janin A, Konttinen YT, Grönblad M, Karhunen P, Gosset D, Malmström M (1990) Fibroblast markers in labial salivary gland biopsies in progressive systemic sclerosis. Clin Exp Rheumatol 8:237–242

Linge C, Green MR, Brooks RF (1989) A method for removal of fibroblasts from human tissue culture systems. Exp Cell Res 185:519–528

Raj NS, Raj CV, Martin J (1984) Selective cytotoxicity of monoclonal antibodies against fibroblasts: application in growth of human corneal epithelial cells in culture. Curr Eye Res 3: 637–643

Saalbach A, Anderegg U, Bruns M, Schnabel E, Herrmann K, Haustein UF (1996) Novel fibroblast-specific monoclonal antibodies: properties and specificities. J Invest Dermatol 106:1314–1319

Singer KH, Sceares RM, Tuck DT, Whichard LP, Denning SM, Haynes BF (1989) Removal of fibroblasts from human epithelial cell cultures with use of a complement fixing monoclonal antibody reactive with human fibroblasts and monocytes/macrophages. J Invest Dermatol 92:166–170

Van Vliet E, Melis M, Foidart JM, Ewijk W van (1986) Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody. J Histochem Cytochem 34:883–890

Weetmann AP, Bright-Thomas R, Freeman M (1990) Regulation of interleukin 6 release by human thyrocytes. J Endocrinol 127: 357–361